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Title page

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JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats

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Abbreviations: BDNF = brain-derived neurotrophic factor; CNS = central nervous system; DRG = dorsal root ganglion; ERK = extracellular signal-regulated protein kinase; GFAP = glial fibrillary acidic protein; IL = interleukin; JAK = Janus kinase; JAK-I = JAK Inhibitor I; JNK = c-jun N-terminal kinase; L5 = fifth lumbar; MMP2 = matrix metalloproteinase 2; NeuN = neuronal nuclei; PBS = phosphate-buffered saline; p-HisH3 = phosphorylated-histone H3; PNI = peripheral nerve injury; STAT3 = signal

transducers and activators of transcription 3; TAK1 = transforming growth factor-activated kinase 1

Neuropathic pain, a debilitating pain condition, is a common consequence of damage to the nervous system. Optimal treatment of neuropathic pain is a major clinical challenge because the underlying mechanisms remain unclear and currently available treatments are frequently ineffective. Emerging lines of evidence indicate that peripheral nerve injury converts resting spinal cord glia into reactive cells that are required for the development and maintenance of neuropathic pain. However, the mechanisms underlying reactive astrogliosis after nerve injury are largely unknown. In the present study, we investigated cell proliferation, a critical process in reactive astrogliosis, and determined the temporally restricted proliferation of dorsal horn astrocytes in rats with spinal nerve injury, a well-known model of neuropathic pain. We found that nerve injury-induced astrocyte proliferation requires the Janus kinase (JAK)-signal transducers and activators of transcription 3 (STAT3) signalling pathway. Nerve injury induced a marked STAT3 nuclear translocation, a primary index of STAT3 activation, in dorsal horn astrocytes. Intrathecally administering inhibitors of JAK-STAT3 signalling to rats with nerve injury reduced the number of proliferating dorsal horn astrocytes and produced a recovery from established tactile allodynia, a cardinal symptom of neuropathic pain that is characterized by

pain hypersensitivity evoked by innocuous stimuli. Moreover, recovery from tactile allodynia was also produced by direct suppression of dividing astrocytes by intrathecal administration of the cell cycle inhibitor flavopiridol to nerve-injured rats. Together, these results imply that the JAK-STAT3 signalling pathway is a critical transducer of astrocyte proliferation and maintenance of tactile allodynia and may be a therapeutic target for neuropathic pain.

Keywords: astrocytes, proliferation, STAT3, neuropathic pain, rats

Introduction

Injury to the nervous system arising from bone compression in cancer, diabetes, infection, autoimmune disease or physical injury results in debilitating chronic pain states (referred to as neuropathic pain) (Baron, 2006). One troublesome hallmark symptom of neuropathic pain is tactile allodynia (pain hypersensitivity evoked by normally innocuous stimuli), which is refractory to currently available treatments, such as non-steroidal anti-inflammatory drugs and even opioids (Scholz and Woolf, 2002; Woolf and Mannion, 1999). Unravelling the molecular and cellular basis for the development and maintenance of pain hypersensitivity after nerve damage is therefore essential for the understanding of mechanisms underlying neuropathic pain and for the development of new therapeutic drugs.

Accumulating evidence from studies utilizing diverse animal models of neuropathic pain indicates that neuropathic pain is a reflection of the aberrant excitability of dorsal horn neurons evoked by peripheral sensory inputs (Costigan et al., 2009; Woolf and Salter, 2000). This hyperexcitability might result from multiple cellular and molecular alterations in the dorsal horn occurring after peripheral nerve injury (PNI). It has long been considered that there are relevant changes in neurons, but many recent studies provide compelling evidence indicating that spinal microglia, immune-like glial cells in

the central nervous system (CNS), rapidly respond to PNI and become activated with changing morphology, increasing their number and expressing a variety of genes (Costigan et al., 2009; Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Scholz and Woolf, 2007; Tsuda et al., 2005; Tsuda et al., 2003; Watkins et al., 2001). Activated spinal microglia secrete various biologically active signalling molecules including proinflammatory cytokines (Inoue, 2006) and brain-derived neurotrophic factor (BDNF) (Trang et al., 2009), which produces hyperexcitability of dorsal horn neurons (Coull et al., 2005; McMahon and Malcangio, 2009).

Compared with rapid progress in our understanding of the microglial regulation of pain, relatively little is known about the role of astrocytes, an abundant cell type in the CNS. However, recent studies have identified signalling molecules in astrocytes that are upregulated by PNI such as extracellular signal-regulated protein kinase (ERK) (Zhuang et al., 2005), c-jun N-terminal kinase (JNK) (Zhuang et al., 2006), transforming growth factor-activated kinase 1 (TAK1) (Katsura et al., 2008), S100β (Tanga et al., 2006) and matrix metalloproteinase 2 (MMP2) (Kawasaki et al., 2008). Importantly, intrathecal administration of inhibitors for these molecules reduces both PNI-induced hyperexcitability of dorsal horn neurons and pain behaviours (Katsura et al., 2008; Kawasaki et al., 2008; Zhuang et al., 2005; Zhuang et al., 2006). Astrocytes receive

signals from activated microglia via interleukin-18 (IL-18), and disrupting this interaction alleviates PNI-induced allodynia (Miyoshi et al., 2008). These molecules are all expressed in reactive astrocytes responding to PNI. In addition, dorsal root injury leads to upregulation of expression of an extracellular serine protease, tissue type plasminogen activator, in spinal reactive astrocytes, and inhibiting the protease reduces root injury-induced mechanical hypersensitivity (Kozai et al., 2007). Therefore, it raises the possibility that the reactive process of dorsal horn astrocytes (reactive astrogliosis) may be crucial for neuropathic pain.

Although a variety of signal transduction pathways have been shown to be involved in the activation of astrocytes *in vitro*, very few signalling modules have been linked to induction of reactive astrogliosis *in vivo* (Sofroniew, 2009; Sofroniew and Vinters, 2010). Reactive astrogliosis is known as a finely graded continuum of progressive cellular and molecular changes in relation to the severity of injury, and is characterized by cellular hypertrophy, hyperplasia, increased glial fibrillary acidic protein (GFAP), proliferation and, in severe cases such as spinal cord injury, scar formation (Sofroniew, 2009; Sofroniew and Vinters, 2010). Among them, proliferation is a critical process for generating numerous reactive astrocytes, which may result in later producing proinflammatory cytokines, thereby modulating dorsal horn pain processing. In contrast

to cellular hypertrophy and GFAP upregulation in dorsal horn astrocytes that have been characterized in various animal models of neuropathic pain (Coyle, 1998; Garrison et al., 1991; Obata et al., 2006; Schwei et al., 1999; Tanga et al., 2004; Vega-Avelaira et al., 2007), the temporal profile and molecular mechanism of proliferation of dorsal horn astrocytes and, more importantly, its role in the pathological process of neuropathic pain remains to be determined.

In the present study, we addressed these issues using the spinal nerve injury model, a well-characterized model of neuropathic pain (Kawasaki et al., 2008; Kim and Chung, 1992; Miyoshi et al., 2008; Tanga et al., 2004; Tsuda et al., 2003; Zhuang et al., 2006). Here, we demonstrate for the first time that (1) temporally restricted proliferation of dorsal horn astrocytes is induced after PNI, (2) it involves the Janus kinase (JAK)-signal transducers and activators of transcription 3 (STAT3) signalling pathway, and (3) intrathecal administration of reagents that inhibit astrocyte proliferation in rats with PNI produces a recovery from tactile allodynia. These results imply that astrocyte proliferation regulated by JAK-STAT3 signalling participates in the maintenance of PNI-induced allodynia.

Materials and Methods

Animals

Male Wistar rats (250-270 g, n=224, Japan SLC, Shizuoka, Japan) were used. Animals were housed in individual cages at a temperature of 22 ± 1°C with a 12-h light-dark cycle (light on 8:30 to 20:30), and fed food and water *ad libitum*. All animal experiments were conducted according to relevant national and international guidelines 'Act on Welfare and Management of Animals' (Ministry of Environment of Japan) and 'Regulation of Laboratory Animals' (Kyushu University), and under the protocols approved by the Institutional Animal Care and Use committee review panels at Kyushu University.

Neuropathic pain model

We used the spinal nerve injury model (Kim and Chung, 1992) with some modifications (Tsuda et al., 2009; Tsuda et al., 2003). Briefly, under isoflurane (2%) anaesthesia, a unilateral fifth lumbar (L5) spinal nerve of rats was tightly ligated by a 5-0 silk suture and cut just distal to the ligature.

Behavioural analysis

Rats were placed individually in a wire mesh cage and habituated for 30-60 min to allow acclimatization to the new environment (Tsuda et al., 2009; Tsuda et al., 2003). Calibrated von Frey filaments (0.4-15 g, Stoelting, IL, USA) were applied to the plantar surface of the rat hindpaw from below the mesh floor. The 50% paw withdrawal threshold was determined using the up-down method (Chaplan et al., 1994). Behavioural measurements were carried out before, 1, 2, 3, 5, 7,10, 14, 17 or 21 days after PNI (see Fig. 6A and 7B). For experiment testing a single administration of AG490 (an inhibitor of the STAT3 activator JAK) on the established allodynia was examined on days 3 and 5 after PNI (Fig. 6D and E) and the behavioural test was performed immediately before 1, 3 and 24 hr after a single bolus injection of AG490 (Fig. 6A). Locomotor activity was measured using a photobeam activity monitoring system (Chronobiology Kit; Stanford Software Systems, Santa Cruz, CA, USA) (Shinohara et al., 2008), and activity counts (number of movements) were recorded for 1 hr.

Drug administration to the intrathecal space

Under isoflurane (2%) anaesthesia, a 32-gauge intrathecal catheter (ReCathCo, PA, USA) was inserted through the atlanto-occipital membrane into the lumbar enlargement

and externalized through the skin (Tsuda et al., 2009). Four days after catheterization, the catheter placement was verified by the observation of hind limb paralysis after intrathecal injection of lidocaine (2%, 5 μl). Animals that failed to display paralysis by lidocaine were not included in the experiments. Two to three days after the lidocaine test, a unilateral L5 spinal nerve of rats was injured as described above. Rats were injected with either AG490 (3 and 10 nmol/10 μl, Calbiochem, CA, USA), JAK Inhibitor I (JAK-I, 2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one, 2.5 nmol/10 μl, Calbiochem) or flavopiridol (5 nmol/10 μl, Santa Cruz Biotechnology, CA, USA) through the catheter once at 9:00 a.m. (AG490 and JAK-I) or twice (once at 9:00 a.m. and once at 19:00 p.m; flavopiridol) a day from day 3 to day 5 (for immunohistochemical experiments of proliferating cells) or to day 7 (for behavioural experiments) (Fig. 5A, 6A and 7B). To examine the role of JAK-STAT3 signalling in tactile allodynia after astrocyte proliferation has finished, PNI rats were intrathecally administered AG490 (10 nmol/10 μl) once a day for 5 days from day 10 post-PNI (Fig. 6A). The behavioural measurements were carried out between 13:00 - 14:00 p.m (Fig. 6A and 7B) (except experiments shown in Fig. 6D and E).

Immunohistochemistry

Rats were deeply anesthetized by intraperitoneal (i.p.) injection of pentobarbital (100 mg/kg) and perfused transcardially with 100 ml of phosphate-buffered saline (PBS; composition in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.5, NaH_2PO_4 8.1; pH 7.4), followed by 250 ml ice-cold 4% (w/v) paraformaldehyde/PBS (time-line: Fig. 5A and 7B). The L5 segments of the spinal cord and dorsal root ganglion (DRG) were removed, post-fixed in the same fixative for 3 hr at 4°C, and placed in 30% (w/v) sucrose solution for 24 hr at 4°C. Transverse spinal cord and DRG sections (30 μm) were incubated in blocking solution (3% (v/v) normal goat serum) for 2 hr at room temperature and then incubated for 48 hr at 4°C with primary antibodies: anti-Ki-67 (rabbit polyclonal, 1:5000, Novocastra, Newcastle, UK), anti-phosphorylated-histone H3 (Ser10) (p-HisH3, rabbit polyclonal, 1:1000, Upstate/Millipore, MA, USA), anti-STAT3 (rabbit polyclonal, 1:1000, Cell Signaling, MA, USA), anti-GFAP (mouse monoclonal, 1:1000, Chemicon, CA, USA), anti-S100β (mouse monoclonal, 1:5000, Sigma-Aldrich), anti-OX-42 (mouse monoclonal, 1:1000, Serotec, Oxford, UK), anti-neuronal nuclei (NeuN, mouse monoclonal, 1:200, Chemicon, CA, USA) and anti-cyclin D1 (rabbit polyclonal, 1:100, TransGenic, Kumamoto, Japan). Following incubation, tissue sections were washed and incubated for 3 hr at room temperature in secondary antibody solution (Alexa Fluor™

488 and/or 546, 1:1000, Molecular Probes, OR, USA). The tissue sections were washed, slide-mounted and subsequently coverslipped with Vectashield hardmount with 4',6'-diamidino-2-phenylindole (DAPI; a cellular nuclear marker, 1.5 μg/ml) (Vector Laboratories, PA, USA). Three to five sections from the L5 spinal cord segments and the L5 DRGs of each rat were randomly selected and were analyzed using an LSM510 Imaging System (Zeiss, Oberkochen, Germany). Fluorescence intensities of STAT3 in ipsilateral and contralateral DRG sections were calculated. The numbers of $GFAP^+/S100\beta^+$ astrocytes and $Iba1^+$ microglia with having clear, visible cell bodies and of p-$HisH3^+$ nuclei with having a small, rounded shape (diameter: approximately 10 μm) and an S/N ratio of 3.0 or more were counted. Background levels were obtained from an area in the dorsal horn of the same section where immunoreactive cells are not contained.

Real-time quantitative RT-PCR

Rats were deeply anesthetized with pentobarbital, perfused transcardially with PBS, and the L4 - 6 spinal cord was removed immediately. Total RNA from the L4 - 6 spinal cord was extracted using Trisure (Bioline, Danwon-Gu, Korea), according to the manufacturer's protocol, and purified using the RNeasy mini plus kit (QIAGEN, CA,

USA). The amount of total RNA was quantified by measuring the OD_{260} using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). For reverse transcription, 200 ng of total RNA was transferred to the reaction with Prime Script reverse transcriptase (Takara, Kyoto, Japan) and random 6 mer primers. Quantitative PCR was carried out with Premix Ex Taq (Takara) using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications, and the data were analyzed by 7500 System SDS Software 1.3.1 (Applied Biosystems) using the standard curve method. All values were normalized with to GAPDH expression. TaqMan probe, forward primer and reverse primer used in this study were designed as follows: STAT3, probe, 5'-FAM-TCGACCTAGAGACCCACTCCTTGCCAG-TAMRA-3'; forward primer, 5'-TTGTGATGCCTCCTTGATTGTC-3', and reverse primer, 5'-ATCGGAGGCTTAGTGAAGAAGTTC-3'; GAPDH, probe, 5'-FAM-ACCACCAACTGCTTAGCCCCCCTG-TAMRA-3'; forward primer, 5'-TGCCCCCATGTTTGTGATG-3'; reverse primer, 5'-GGCATGGACTGTGGTCATGA-3'.

Statistics. Statistical analyses of results were evaluated using the unpaired Student's *t* test, the Mann-Whitney *U* test or the Freedman test. Analysis of the time-course of PNI-induced tactile allodynia between vehicle- and drug-treated groups was performed by two factors (group & times) repeated measures analysis of variance (ANOVA). Values were considered significantly different at $P < 0.05$.

Results

Proliferation activity of dorsal horn astrocytes after PNI

To visualize proliferating cells in the L5 dorsal horn after PNI induced by injury of the L5 spinal nerve, we performed immunohistochemical experiments using Ki-67, a nuclear protein expressed in all phases of the cell cycle except the resting phase (Taupin, 2007). In contrast to normal rats that only had very few Ki-67-positive (Ki-67^+) cells in the dorsal horn, 2 and 5 days after PNI a number of strong Ki-67^+ cells were observed in the dorsal horn ipsilateral to the injury (Fig. 1). Ki-67^+ cells were still observed 10 days post-PNI, but the number of Ki-67^+ cells markedly decreased (Fig. 1). To identify the type of cells positive for Ki-67, we performed double-immunolabelling for Ki-67 and for cell-type-specific markers. Almost all Ki-67^+ cells in the dorsal horn 2 days

post-injury were double-labelled with OX-42 (a microglia marker), but not with GFAP (an astrocyte marker) or NeuN (a neuronal marker) (Fig. 1B). This result is consistent with our (Inoue and Tsuda, 2009) and other previous reports (Echeverry et al., 2008; Suter et al., 2007) showing microglial proliferation around 2 days after PNI. Unexpectedly, on day 5, we found that cells with Ki-67 immunoreactivity were not double-labelled for OX-42 or NeuN. Instead, almost all Ki-67$^+$ cells were double-labelled with GFAP (Fig. 1B), indicating proliferation of astrocytes in the dorsal horn after PNI.

To determine the mitotic phase of cycling astrocytes, we immunostained for phosphorylated-histone H3 (p-HisH3), a marker for the G_2/M phase of the cell cycle (Hendzel et al., 1997; Taupin, 2007). On postoperative day 5, strong p-HisH3$^+$ cells were seen in the dorsal horn ipsilateral to the PNI but not in the contralateral dorsal horn (Fig. 2A) or in the dorsal horn of control rats (data not shown). Double-immunolabelling experiments using cell markers revealed that GFAP$^+$ cells expressed p-HisH3 immunofluorescence (Fig. 2B and C). By counting p-HisH3$^+$ cells in the grey matter of the dorsal horn, we found a marked increase in the number of p-HisH3$^+$ cells on day 5 as well as day 2 post-PNI (P<0.05, Fig. 2D). Consistent with our data using Ki-67, on day 2, 86% of total p-HisH3$^+$ cells were positive for the

microglial marker OX-42, and, conversely, on day 5 a substantial proportion of p-HisH3$^+$ dividing cells were identified as astrocytes following double-labelling with GFAP (86 % of total p-HisH3$^+$ cells) (Fig. 2D). The number of cells that showed p-HisH3$^+$/OX-42$^+$ and p-HisH3$^+$/GFAP$^+$ were significantly different (day 2, $P<0.05$; day 5, $P<0.01$). We further quantified the number of p-HisH3$^+$/GFAP$^+$ cells in the dorsal horn at 12-hr intervals from postoperative day 2. A marked increase in p-HisH3$^+$/GFAP$^+$ dividing cells started from 4 days after PNI, peaked on day 5 and then returned to the pre-injured level over the next 5 days (10 days post-injury) (Fig. 2E). No significant change in the number of p-HisH3$^+$/GFAP$^+$ cells was observed at days 10 and 14 (Fig. 2E). In addition, we confirmed a significant increase in the number of astrocytes (GFAP$^+$/S100β$^+$) in the ipsilateral dorsal horn on postoperative day 7 (see below) and days 14 and 21 (Supplementary Fig. 1). These results imply a shift of actively-cycling cells from microglia to astrocytes in the dorsal horn after PNI, and we identified astrocytes as the principal type of dividing cell in the dorsal horn from day 4 to day 7 post-PNI.

Role of STAT3 in astrocyte proliferation in the dorsal horn after PNI

To explore the mechanisms regulating astrocyte proliferation, we investigated the role of STAT3 signalling. STAT3 is a principal mediator in a variety of biological processes, including cell proliferation (Levy and Lee, 2002), and there is evidence that JAK-STAT3 signalling regulates proliferation of cultured astrocytes *in vitro* (Sarafian et al., 2010; Washburn and Neary, 2006). Quantitative PCR analysis demonstrated that the level of STAT3 mRNA in the spinal cord was significantly increased in the ipsilateral side on day 5 ($P<0.05$, Fig. 3A). By immunostaining L5 dorsal horn sections with a STAT3 antibody, we observed punctate STAT3 immunofluorescence dotted in the grey matter of the dorsal horn 5 days after PNI compared with the contralateral side (Fig. 3B and C). The STAT3 immunofluorescence was evident from post-PNI day 3 (Fig. 3C). By contrast, STAT3 expression in the DRG was significantly decreased in the ipsilateral DRG on day 5 postPNI ($P<0.001$, Fig. 3D and E).

To define the cells expressing punctate STAT3 immunofluorescence, we performed double-immunolabelling with cell type markers and found that almost all $STAT3^+$ cells were double-labelled with GFAP (Fig. 4A and B) and S100β (Fig. 4C), both of which are markers of astrocytes, but not with OX-42 and NeuN (Fig. 4C). STAT3 immunofluorescence accumulated in the nuclear region that was stained by DAPI (Fig.

4E). Active STAT3 is known to translocate to the nucleus (Reich and Liu, 2006), suggesting that dorsal horn astrocytes express activated STAT3 after PNI.

To investigate the role of STAT3 in astrocyte proliferation *in vivo*, we administered AG490, an inhibitor of the STAT3 activator JAK, intrathecally once a day for 2 days from day 3 after PNI (Fig. 5A). AG490 reduced the PNI-induced STAT3 translocation in $GFAP^+$ astrocytes in the dorsal horn (Fig. 5B). On day 5, the number of p-$HisH3^+$/$GFAP^+$ cells in the dorsal horn was significantly lower in AG490-treated rats than in vehicle-treated rats ($P<0.01$, Fig. 5C and D). A similar result was obtained from PNI rats treated with a JAK inhibitor (JAK Inhibitor I: JAK-I) ($P<0.05$, Fig. 5D). In addition, AG490 or JAK-I treatment resulted in a decrease in GFAP immunofluorescence in the dorsal horn 7 days after PNI (Fig. 5E and F).

Effect of STAT3 inhibition on nerve injury-induced tactile allodynia

To examine the role of astrocyte proliferation in PNI-induced tactile allodynia, we measured paw withdrawal threshold to mechanical stimulation. PNI rats (treated with vehicle) displayed a marked decrease in the paw withdrawal threshold of the ipsilateral side ($F_{6,139}=6.165$, $P<0.001$) but not contralateral side (Fig. 6B). PNI rats treated with AG490 (10 nmol) from day 3 to 7 (Fig. 6A), a regimen based on the time course of

astrocyte cycling (Fig. 2E), showed a significant recovery in the decreased paw withdrawal threshold ($F_{6,143}$=12.747, P<0.001; day 5, P<0.05; day 7, P<0.01; Fig. 6B). A similar recovery from allodynia on day 7 was observed in PNI rats treated with either AG490 (3 nmol; P<0.01) or JAK-I (2.5 nmol; P<0.05) (Fig. 6C). After the last administration of AG490 on day 7, paw withdrawal threshold still remained elevated on day 10 (P<0.001, Fig. 6B). We tested the effect of acute inhibition of STAT3 signalling by a single bolus intrathecal injection of AG490 (10 nmol) on postoperative day 3 or 5. AG490 did not produce any effect on paw withdrawal threshold over a period of 24 hr (Fig. 6D and 6E). Notably, neither motor abnormality nor sedative effects were observed in either AG490- or JAK-I-treated rats on day 7 [locomotor activity (counts for 1 hr): vehicle, 604.3 ± 83.8 (n=7); AG490, 596.3 ± 203.5 (n=4); JAK-I, 774.3 ± 105.7 (n=3)]. These results indicate that inhibiting the JAK-STAT3 signalling pathway suppresses both proliferation of dorsal horn astrocytes and the maintenance of tactile allodynia. We also examined the role of JAK-STAT3 signalling after astrocyte proliferation had finished. Intrathecal administration of AG490 for 5 days from day 10 post-PNI (Fig. 6A) also significantly reduced the established tactile allodynia ($F_{4,15}$=2.794, P<0.05; days 12 and 14, P<0.05, Fig. 6F). The anti-allodynic effect of AG490 (Fig. 6F) was weaker than that of AG490 administered from day 3 post-PNI

(Fig. 6B), and after the last administration of AG490 on day 14, the paw withdrawal threshold on day 17 returned to the pre-injection level (Fig. 6F).

Effects of the cyclin-dependent kinase inhibitor flavopiridol on astrocyte proliferation and tactile allodynia

If astrocyte proliferation contributes to neuropathic pain, then interfering with astrocyte cycling should alleviate allodynia. To test this hypothesis, we conducted an independent test using the cell cycle inhibitor flavopiridol that inhibits astrocyte proliferation *in vitro* and *in vivo* (Byrnes et al., 2007; Di Giovanni et al., 2005). Flavopiridol inhibits cyclin-dependent kinases (CDKs), leads to a reduction in cyclin D1 expression, and arrests cells in G_1 or at the G_2/M transition (Swanton, 2004). Because cyclin D1 is essential for astrocyte proliferation (Zhu et al., 2007), we first examined cyclin D1 expression in the dorsal horn. The expression of cyclin D1 was induced in the dorsal horn 5 days post-injury (Fig. 7A), and almost all cyclin $D1^+$ cells were double-labelled with GFAP (98.6 % of total cyclin $D1^+$ cells; Fig. 7A). We administered flavopiridol (5 nmol) intrathecally to PNI rats twice a day for 2 days from postoperative day 3 (Fig. 7B). The number of p-$HisH3^+$/$GFAP^+$ cells in the dorsal horn on day 5 was significantly lower in flavopiridol-treated rats than vehicle-treated rats

(P<0.01, Fig. 7C and D). Furthermore, flavopiridol also significantly reduced the PNI-induced the increase in the number of $GFAP^+/S100\beta^+$ astrocytes in the dorsal horn on day 7 (P<0.01, Fig. 7E) without affecting that of $Iba1^+$ microglia (Fig. 7F). Behaviourally, PNI rats treated with flavopiridol (5 nmol) from day 3 to 7 showed a significant recovery in the decreased paw withdrawal threshold after the injury ($F_{6,44}$=3.19, P<0.01; day 5, P<0.01; day 7, P<0.001; Fig. 7G). After the last administration of flavopiridol on day 7, the significant attenuation of decreased paw withdrawal threshold remained on day 10 (P<0.05, Fig. 7G).

Discussion

A rapidly growing body of evidence has indicated that reactive spinal astrocytes, as well as microglia, are critical components for maintaining neuropathic pain (Costigan et al., 2009; Hald, 2009; Inoue and Tsuda, 2009; Marchand et al., 2005; McMahon and Malcangio, 2009; Milligan and Watkins, 2009; Scholz and Woolf, 2007; Suter et al., 2007; Tsuda et al., 2005; Watkins et al., 2001). Despite such recent progress, very little is known about the reactive process of astrocytes in the dorsal horn after PNI, in particular cell proliferation, a critical process in reactive astrogliosis. In the present

study, our detailed immunohistochemical analyses utilizing three independent markers of the cell cycle (Ki-67, p-HisH3 and cyclin D1) now provide compelling evidence that dorsal horn astrocytes undergo proliferation after PNI in rats. Consistent with previous observations using 5-bromo-2'-deoxyuridine (BrdU) (Echeverry et al., 2008; Narita et al., 2006; Suter et al., 2007), these three markers also successfully detected proliferating microglia 2 days post-PNI, confirming the specificity of these markers. Apparently, our findings are not in line with a prevailing view that dorsal horn astrocytes do not proliferate after PNI (Echeverry et al., 2008; Gehrmann and Banati, 1995; Hald, 2009; Suter et al., 2007). However, this might be explained by the following reasons. First, these previous studies did not test proliferation activity around day 5 post-PNI. Second, only the S-phase marker BrdU, whose half-life is very short (Taupin, 2007), was used for detecting proliferating cells. Third, the present study used Ki-67 that labels cells in all phases of the cell cycle except the resting phase (Taupin, 2007). Furthermore, by detecting p-HisH3^{+}/GFAP^{+} astrocytes at 12-hr intervals, we could determine the temporally restricted proliferation activity of dorsal horn astrocytes after PNI. To our knowledge, this is the first report determining the time course of astrocyte proliferation in the dorsal horn in a model of neuropathic pain and provides evidence for changing the prevailing view.

By showing STAT3 nuclear translocation (a primary index of activation) restricted to astrocytes in the dorsal horn after PNI and the suppression of dividing astrocytes by JAK inhibitors, our findings further demonstrate that PNI-induced astrocyte proliferation in the rat dorsal horn is regulated by the JAK-STAT3 signalling pathway. Astrocytic STAT3 activation has also been observed under other neuropathological conditions accompanied by reactive astrogliosis, such as spinal cord injury (Herrmann et al., 2008; Okada et al., 2006), brain ischemia (Choi et al., 2003), dopamine neuron damage in the striatum (Sriram et al., 2004) and facial nerve axotomy (Schwaiger et al., 2000). However, there is conflicting reports that immunofluorescence of phosphorylated STAT3 increases in spinal microglia 24 hr after PNI and, to a much lesser extent, 7 days after PNI (Dominguez et al., 2010; Dominguez et al., 2008). A striking difference is the subcellular localization of STAT3. In their studies, immunofluorescence of phospho-STAT3 is not localized in the nucleus but is merged with OX-42 immunofluorescence, a cell surface marker of microglia. By contrast, our data clearly shows the nuclear translocation of STAT3 in an astrocyte-specific manner, which is demonstrated by using two astrocyte markers and the nuclear marker DAPI. Although it is uncertain whether phosphorylated STAT3 in the cytoplasm of microglia functions as a transcription factor, STAT3's function in dorsal horn astrocytes would be different in

microglia. The present study shows that proliferating dorsal horn astrocytes are suppressed by spinal administration of the JAK inhibitors AG490 and JAK-I during the period when astrocyte-restricted proliferation and STAT3 activation occur. Thus, it is reasonable to conclude that inhibiting JAK-STAT3 signalling in dorsal horn astrocytes results in suppression of their proliferation. However, we can not exclude the possible involvement of STAT3 in the DRG to which drugs administered intrathecally can reach (Zhuang et al., 2006). Nevertheless, STAT3 expression markedly decreased in the injured DRG, and, importantly, recent studies have demonstrated impaired proliferation activity of either AG490-treated or STAT3-deficient cultured astrocytes (Sarafian et al., 2010; Washburn and Neary, 2006). These results provide direct evidence for a crucial role of astrocytic JAK-STAT3 signalling in their proliferation and strongly support our conclusion. It should also be noted that dorsal horn astrocyte proliferation after PNI was not completely abolished by AG490 and JAK-I, suggesting that there may be independent and/or cooperative mechanisms involving other signals. ERK and JNK are known to regulate astrocyte proliferation *in vitro* (Gadea et al., 2008; Neary et al., 1999). After PNI, ERK and JNK are activated in dorsal horn astrocytes (Zhuang et al., 2005; Zhuang et al., 2006). However, astrocytic ERK activation occurs from 10 days after PNI (Zhuang et al., 2005). Astrocytic JNK is gradually activated from day 3

post-PNI, but a JNK inhibitor fails to suppress the number of $GFAP^+$ dorsal horn astrocytes (Zhuang et al., 2006). Thus, it is unlikely that these kinases contribute to the proliferation of dorsal horn astrocytes after PNI *in vivo*.

The mechanisms underlying STAT3-mediated proliferation of dorsal horn astrocytes remain unknown. STAT3 nuclear translocation may induce transcriptional changes in astrocytes, leading to the proliferation of these cells. There are a number of genes whose expression is controlled by STAT3 in cultured astrocytes, with an estimation over 1200 (Sarafian et al., 2010). Among genes encoding cell cycle proteins, the well-known cell cycle driver cyclin D1 is increased by STAT3 (Sarafian et al., 2010). Our findings demonstrate that the expression of cyclin D1 protein in the dorsal horn is induced specifically in astrocytes after PNI, and, interestingly, cyclin D1-deficient mice have been reported to display impaired proliferation activity of astrocytes *in vivo* (Zhu et al., 2007). Thus, it is possible that cyclin D1 may participate in STAT3-dependent dorsal horn astrocyte proliferation within a specific time window after PNI.

As very few extracellular signalling molecules have been linked to reactive astrogliosis in the dorsal horn after PNI *in vivo*, our findings demonstrating JAK-STAT3 signalling as a critical pathway of dorsal horn astrocyte proliferation

would aid future investigations to identify molecule(s) required for PNI-induced reactive astrogliosis. It has been reported that PNI results in upregulation of signalling molecules, which can activate STAT3, in the DRG and spinal cord, including IL-6 (Murphy et al., 1999) and fibroblast growth factor-2 (FGF-2) (Ji et al., 2006; Madiai et al., 2005). Activator for κ-opioid receptor (KOR) may also be involved (Xu et al., 2007). It is also interesting to note that proliferation of dorsal horn astrocytes after PNI occurs after that of microglia. Identification of factors that are responsible for STAT3-dependent astrocyte proliferation and their cellular source is an important issue that needs to be investigated in future studies.

Another important finding in the present study was that suppression of astrocyte proliferation by inhibitors of JAK-STAT3 signalling leads to a recovery of tactile allodynia, a major feature of PNI-induced neuropathic pain. Although the anti-allodynic effect of AG490 when administered before PNI has previously been reported (Dominguez et al., 2008; Maeda et al., 2009), our findings reveal the first evidence that AG490 and JAK-I effectively alleviate tactile allodynia even by posttreatment with the inhibitors after PNI. Inhibition of tactile allodynia by intrathecal administration of AG490 and JAK-I from day 3 to 7, a time window corresponding with astrocyte proliferation, implies that recovery from allodynia is associated with the reduction of

proliferating astrocytes. In support of this, recovery from tactile allodynia was mimicked by direct suppression of dividing astrocytes by the cell cycle inhibitor flavopiridol. Although astrocyte proliferation was inhibited equally by JAK inhibitors and flavopiridol, it appears that JAK inhibitors were more effective than flavopiridol in attenuating allodynia. Besides proliferation, STAT3 also regulates reactive astrogliosis such as cellular hypertrophy and GFAP expression (Sofroniew, 2009). Moreover, STAT3 controls transcription of MMP-2 (Xie et al., 2004) and monocyte chemoattractant protein-1 (Potula et al., 2009), critical molecules that have been shown to be upregulated in reactive astrocytes and implicated in the genesis of neuropathic pain (Gao et al., 2009; Kawasaki et al., 2008). Indeed, AG490 administered after astrocyte proliferation had finished produced a partial recovery of tactile allodynia. Thus, it is likely that inhibition of JAK-STAT3 signalling may globally repress reactive astrogliosis by inhibiting PNI-elicited astrocyte proliferation and gene expression in reactive astrocytes in the dorsal horn.

In summary, our findings here support the idea that reactive astrocytes contribute to maintaining neuropathic pain. In addition, we show that astrocytic JAK-STAT3 signalling is important for regulating astrocyte proliferation and that disrupting this proliferative process alleviates neuropathic pain after PNI. The levels of STAT3

immunofluorescence accumulated in the nuclear region were much lower in the dorsal horn of naive rats and in the contralateral dorsal horn of PNI rats as compared with the ipsilateral dorsal horn of PNI rats. The JAK-STAT3 signalling pathway serves as a target for pharmacological modification of reactive astrogliosis in the dorsal horn responding to PNI and for treating neuropathic pain. In addition, allodynia is also known as a severe side effect after neural stem cell transplantation, which is associated with differentiation to astrocytes (Hofstetter et al., 2005). Interestingly, STAT3 signalling is implicated in astrogliogenesis from neural stem cells (Bonni et al., 1997). Thus, targeting JAK-STAT3 signalling may also prevent the side effects of neural stem cell therapy and thereby improve its efficacy.

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Figure legends

Fig. 1 Immunohistochemical characterization of proliferating cells in the dorsal horn after PNI in rats. (**A**) Immunofluorescence of Ki-67, a nuclear protein expressed in all cell cycle phases except the resting phase, in L5 dorsal horn sections on day 0, 2, 5 and 10 after PNI. (**B**) Double immunofluorescence labelling for Ki-67 (green) and cell-type markers (magenta: OX-42, microglia; GFAP, astrocytes; NeuN, neurons) in L5 dorsal horn sections on day 2 (upper three panels) and day 5 (lower three panels) post-PNI. Scale bar: 200 μm (**A**), 50 μm (**B**).

Fig. 2 Mitotic phase of cycling astrocytes in the dorsal horn after PNI in rats. (**A**) Immunofluorescence of p-HisH3, a marker for G_2/M phase of the cell cycle, in L5 dorsal horn sections 5 days after PNI. (**B**) Double immunofluorescence labelling for p-HisH3 (green) and GFAP (magenta) shown in a single channel (p-HisH3, left) and as a merged image (p-HisH3/GFAP, right) from grey matter of the L5 dorsal horn 5 days after PNI. (**C**) Representative confocal *z*-stack digital images of a single cell double-immunolabelled with p-HisH3 (green) and GFAP (magenta). (**D**) The numbers of p-HisH3$^+$ cells (open and closed columns), p-HisH3$^+$/OX-42$^+$ cells (yellow columns) and p-HisH3$^+$/GFAP$^+$ cells (red columns) in L5 dorsal horn sections from rats 0, 2 and 5

days after PNI. Values represent the number of cells (per dorsal horn) (n=4-6 rats; *P<0.05, **P<0.01). (**E**) The time course of p-HisH3$^+$/GFAP$^+$ cells in the dorsal horn after PNI. Values represent the number of p-HisH3$^+$/GFAP$^+$ cells (per dorsal horn) (n=3-5 rats per each time point; *P<0.05, **P<0.01 vs. contralateral side at the corresponding time point). Scale bar: 200 μm (**A**), 50 μm (**B**), 10 μm (**C**). Data are mean ± SEM.

Fig. 3 STAT3 expression in the dorsal horn and DRG after PNI in rats. (**A**) Real-time quantitative RT-PCR analysis of STAT3 mRNA in the total RNA extract from the rat spinal cord ipsilateral and contralateral to the PNI on day 0 and 5 after PNI. The levels of STAT3 mRNA were normalized to the value of GAPDH mRNA, and values represent the ratio of STAT3 mRNA/GAPDH mRNA (n=4 rats; *P<0.05, **P<0.01 vs. contralateral side at the corresponding time point). (**B**) Immunofluorescence of STAT3 in L5 dorsal horn sections 5 days after PNI. (**C**) STAT3 immunofluorescence images at high-magnification from grey matter of the L5 dorsal horn 0, 3 and 5 days after PNI. (**D**) Immunofluorescence of STAT3 in L5 DRG sections 5 days after PNI. (E) Fluorescence intensity of STAT3 in the L5 DRG on day 5 post-PNI (n=3 rats; ***P<0.001 vs. contralateral side). Scale bar: 200 μm (**B**), 50 μm (**C**, **D**). Data are mean

± SEM.

Fig. 4 STAT3 translocation to the nucleus of dorsal horn astrocytes after PNI in rats. (**A**-**D**) Double immunofluorescence labelling for STAT3 (green) and cell-type markers (magenta; **A**, GFAP; **B**, S100β; **C**, OX-42; **D**, NeuN) in L5 dorsal horn sections on day 5 post-PNI. (**E**) Representative confocal *z*-stack digital images of a single cell triple-labelled with STAT3 (green), GFAP (magenta) and DAPI (blue) shown in double channels (left, STAT3/GFAP; middle, DAPI/GFAP) and as a merged image (right, STAT3/GFAP/DAPI) from grey matter of the L5 dorsal horn 5 days after PNI. Scale bar: 50 μm (**A**-**D**), 10 μm (**E**).

Fig. 5 Role of STAT3 signalling in dorsal horn astrocyte proliferation after PNI. (**A**) Schematic time-line for intrathecal administration and fixation. IHC, immunohistochemistry. AG490 and JAK-I, respective inhibitors of the STAT3 activator JAK, were administered intrathecally once a day for 2 days (**B**, **C, D**) and for 5 days (**E, F**) from day 3 after PNI. (**B**) STAT3 immunofluorescence in representative images of $GFAP^+$ cells in the L5 dorsal horn sections from vehicle-treated and AG490-treated rats. (**C**) p-HisH3 immunofluorescence in representative images of L5 dorsal horn sections

from vehicle-treated and AG490-treated rats. (**D**) The numbers of p-HisH3^{+}/GFAP^{+} cells in the L5 dorsal horn ipsilateral and contralateral to PNI from vehicle-, AG490- and JAK-I-treated rats 5 days post-PNI. Values represent the number of p-HisH3^{+}/GFAP^{+} cells (per dorsal horn) (*n*=3-7 rats; ****P*<0.001 vs. contralateral side of vehicle group; #*P*<0.05, ##*P*<0.01 vs. ipsilateral side of vehicle group). (**E, F**) GFAP immunofluorescence in representative images of L5 dorsal horn sections from PNI rats treated with either vehicle, AG490 or JAK-I on postoperative day 7. Scale bar: 10 μm (**B**), 200 μm (**C, E, F**). Data are mean ± SEM.

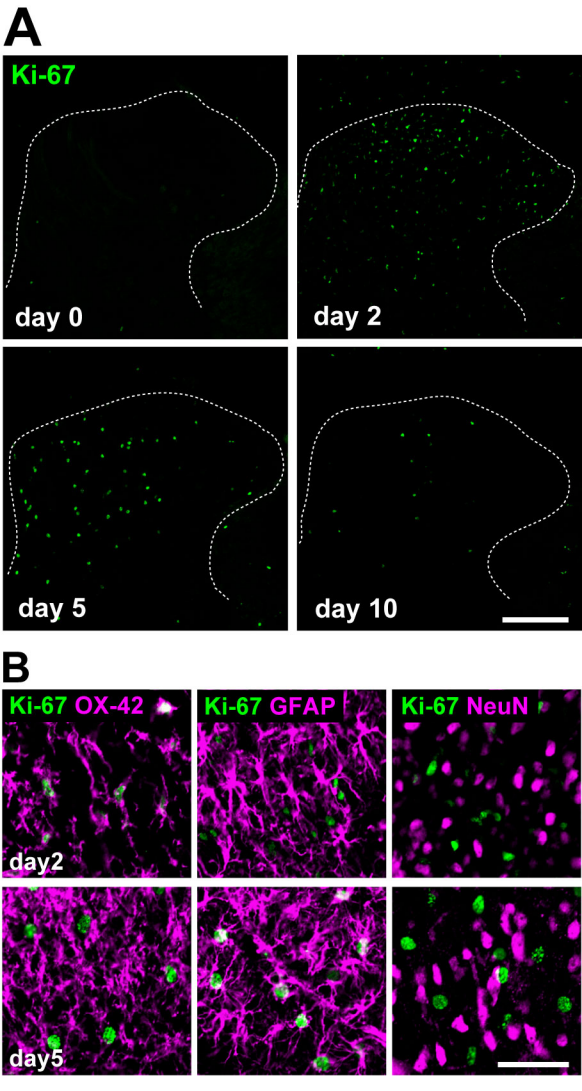
Fig. 6 Recovery from tactile allodynia after PNI by inhibiting JAK-STAT3 signalling in rats. (**A**) Schematic time-line for intrathecal administration and behavioural tests. (**B**) PNI rats were injected intrathecally with AG490 (10 nmol/10 μl) or vehicle (10 μl) once a day for 5 days from day 3 to day 7. Paw withdrawal threshold to mechanical stimulation by von Frey filaments was measured before (day 0), 1, 2, 3, 5, 7 and 10 days after PNI. Values represent the threshold (g) to elicit paw withdrawal behaviour (PWT) (*n*=5 rats; **P*<0.05, ***P*<0.01, ****P*<0.001 vs. vehicle group at corresponding time point). (**C**) Paw withdrawal threshold on day 7 in PNI rats treated intrathecally with AG490 (3 or 10 nmol/10 μl), JAK-I (2.5 nmol/10 μl) or vehicle (10 μl) once a day for 5

days from day 3 to day 7. Values represent the threshold (g) to elicit paw withdrawal behaviour (PWT) (n=5-7 rats; *P<0.05, **P<0.01 vs. vehicle group). (**D, E**) Paw withdrawal threshold on day 3 (**D**) and day 5 (**E**) in PNI rats with a single bolus intrathecal injection of AG490 (10 nmol/10 μl) or vehicle (10 μl). PWT was measured before (day 0), pre-injection (**D**, day 3, 0 hr; **E**, day 5, 0 hr), 1, 3 and 24 hr after injection. Values represent the threshold (g) to elicit paw withdrawal behaviour (PWT) (n=4-6 rats). (**F**) PNI rats were injected intrathecally with AG490 (10 nmol/10 μl) or vehicle (10 μl) once a day for 5 days from day 10 to day 14. Paw withdrawal threshold was measured 10, 12, 14, 17 and 21 days after PNI. Values represent the threshold (g) to elicit paw withdrawal behaviour (PWT) (n=5 rats; *P<0.05 vs. vehicle group at corresponding time point). Data are mean ± SEM.

Fig. 7 Effects of the cell cycle inhibitor flavopiridol on astrocyte proliferation and tactile allodynia after PNI. (**A**) Double immunofluorescence labelling for cyclin D1 (green) and GFAP (magenta) shown as a merged image from grey matter of the L5 dorsal horn 5 days after PNI. The numbers of cyclin $D1^+$ cells (open and closed columns), cyclin $D1^+/GFAP^+$ cells (green column) and cyclin $D1^+/GFAP^-$ cells (dark green column) in L5 dorsal horn sections from rats 5 days after PNI. Values represent

the number of cells (per section) (n=4 rats; *P<0.05). (**B**) Schematic time-line for intrathecal administration, fixation and behavioural tests. IHC, immunohistochemistry. (**C**) p-HisH3 immunofluorescence in representative images of L5 dorsal horn sections from vehicle- and flavopiridol-treated rats on day 5 post-PNI. PNI rats were injected intrathecally with flavopiridol (5 nmol/10 μl) or vehicle (10 μl) twice a day for 2 days from day 3. (**D**) The numbers of p-HisH3$^+$/GFAP$^+$ cells in the L5 dorsal horn ipsilateral and contralateral to PNI from vehicle- or flavopiridol-treated rats on 5 days post-PNI. Values represent the number of p-HisH3$^+$/GFAP$^+$ cells (per section) (n=5 rats; ***P<0.001 vs. contralateral side of vehicle group; ##P<0.01 vs. ipsilateral side of vehicle group). (**E, F**) The numbers of GFAP$^+$/S100β$^+$ (**E**) and Iba1$^+$ (**F**) cells in the L5 dorsal horn ipsilateral and contralateral to PNI from vehicle- or flavopiridol-treated rats on day 7 post-PNI. Values represent the number of GFAP$^+$/S100β$^+$ (**E**) and Iba1$^+$ (**F**) cells (per section) (n=4 rats; ***P<0.001 vs. contralateral side of vehicle group; ##P<0.01 vs. ipsilateral side of vehicle group). (**G**) Paw withdrawal threshold to mechanical stimulation by von Frey filaments was measured before (day 0), and 1, 3, 5, 7 and 10 days after PNI. PNI rats were injected intrathecally with flavopiridol (5 nmol/10 μl) or vehicle (10 μl) twice a day for 5 days from day 3 to day 7. Values represent the threshold (g) to elicit paw withdrawal behaviour (PWT) (n=5 rats;

*P<0.05, **P<0.01, ***P<0.001 vs. vehicle group at corresponding time point). Scale bar: 50 μm (**A**), 200 μm (**C**). Data are mean ± SEM.



Revised Figure 1
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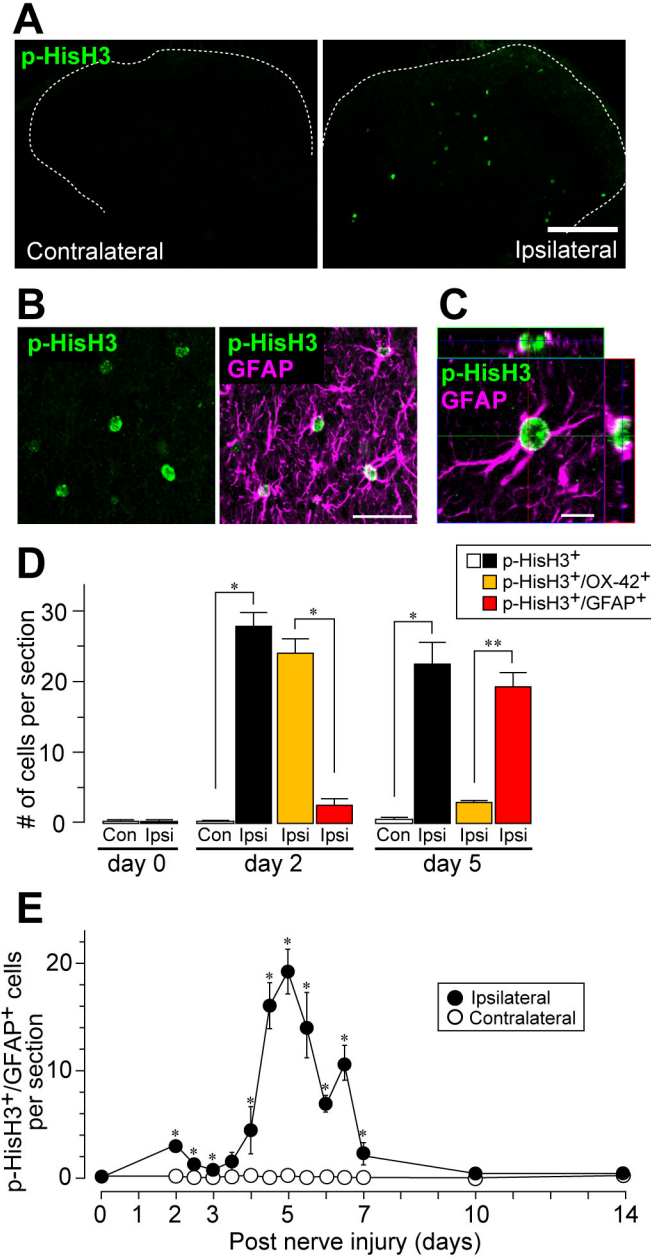

Revised Figure 2
BRAIN-2010-01243
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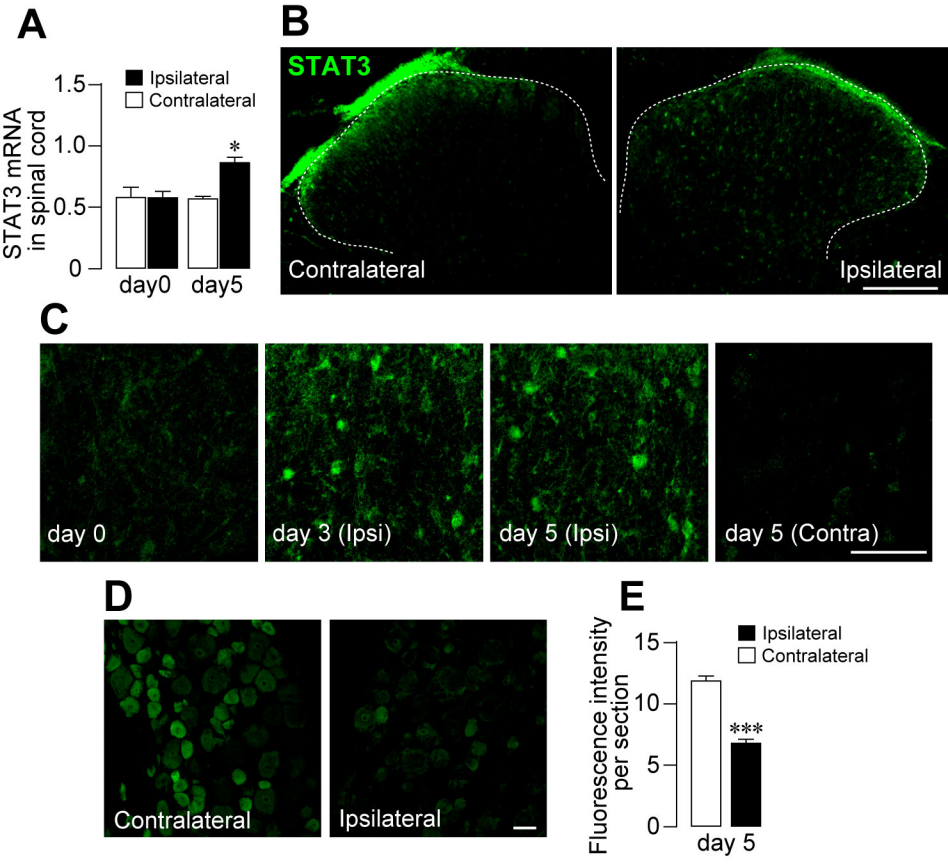

Revised Figure 3
BRAIN-2010-01243
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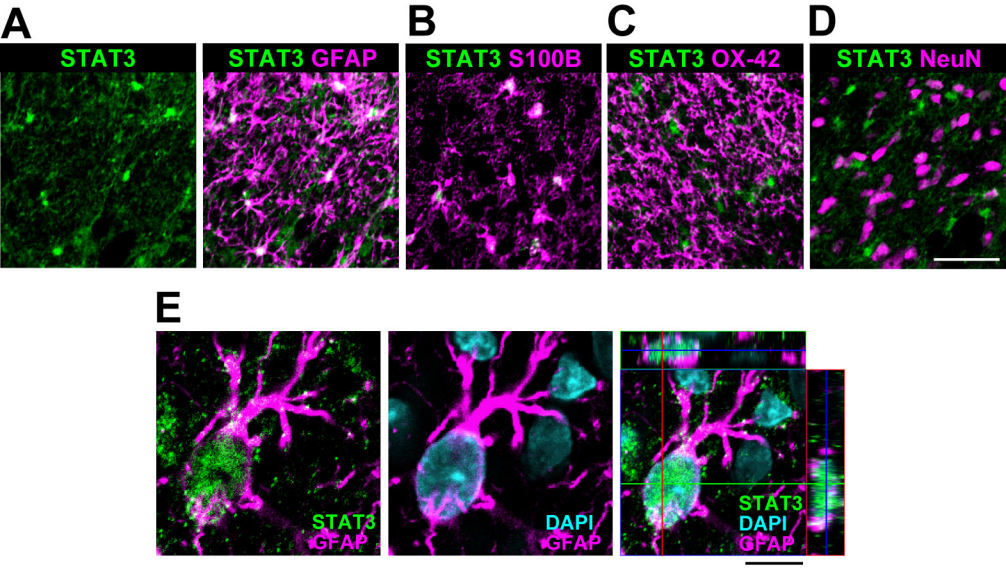

Revised Figure 4
BRAIN-2010-01243
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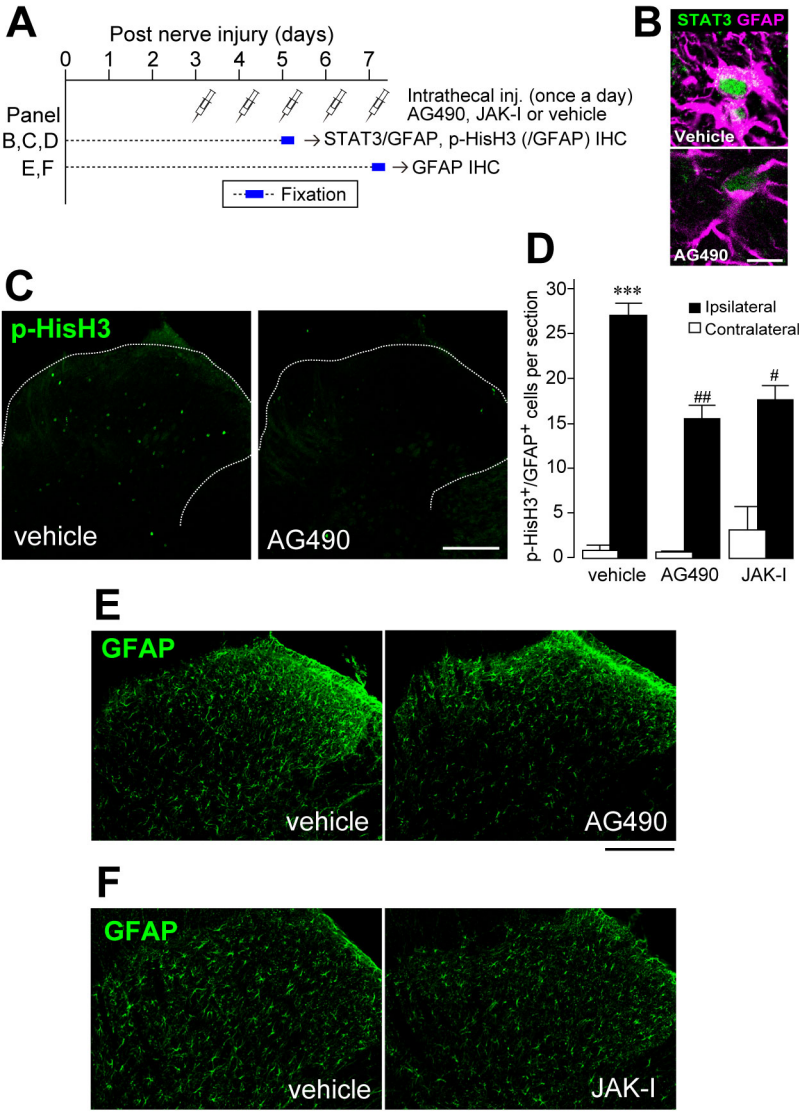

Revised Figure 5
BRAIN-2010-01243
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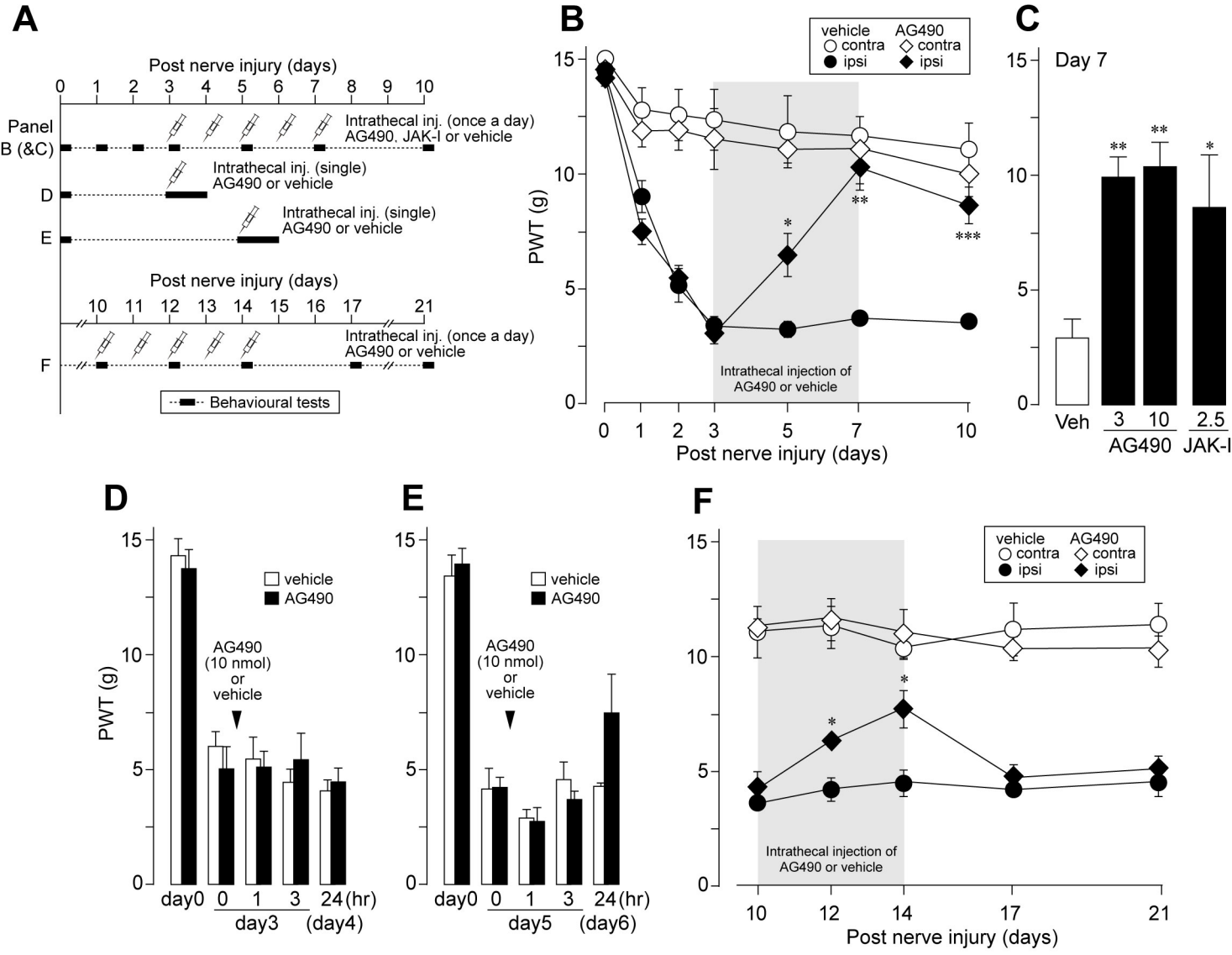

Revised Figure 6
BRAIN-2010-01243
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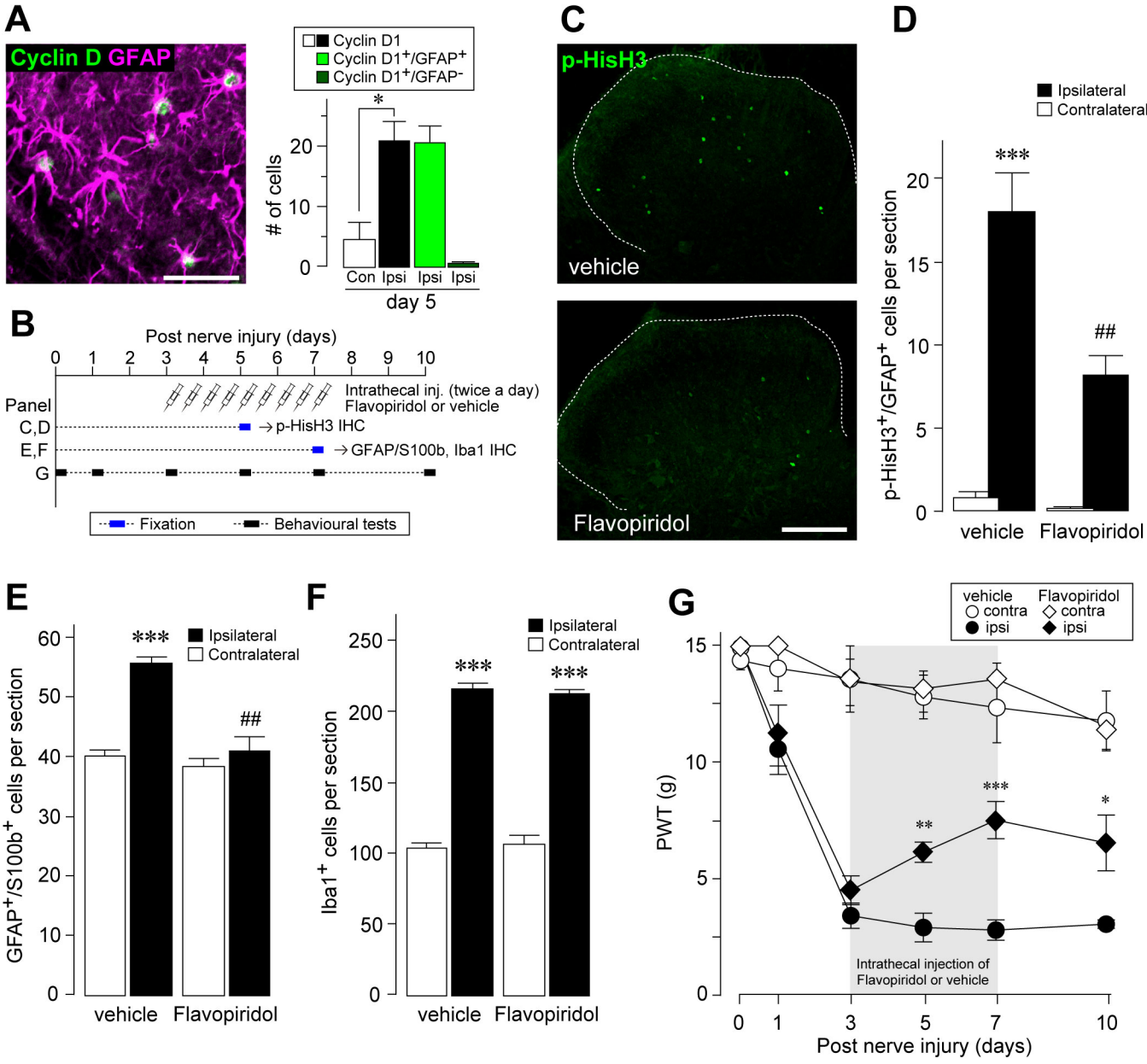

Revised Figure 7
BRAIN-2010-01243
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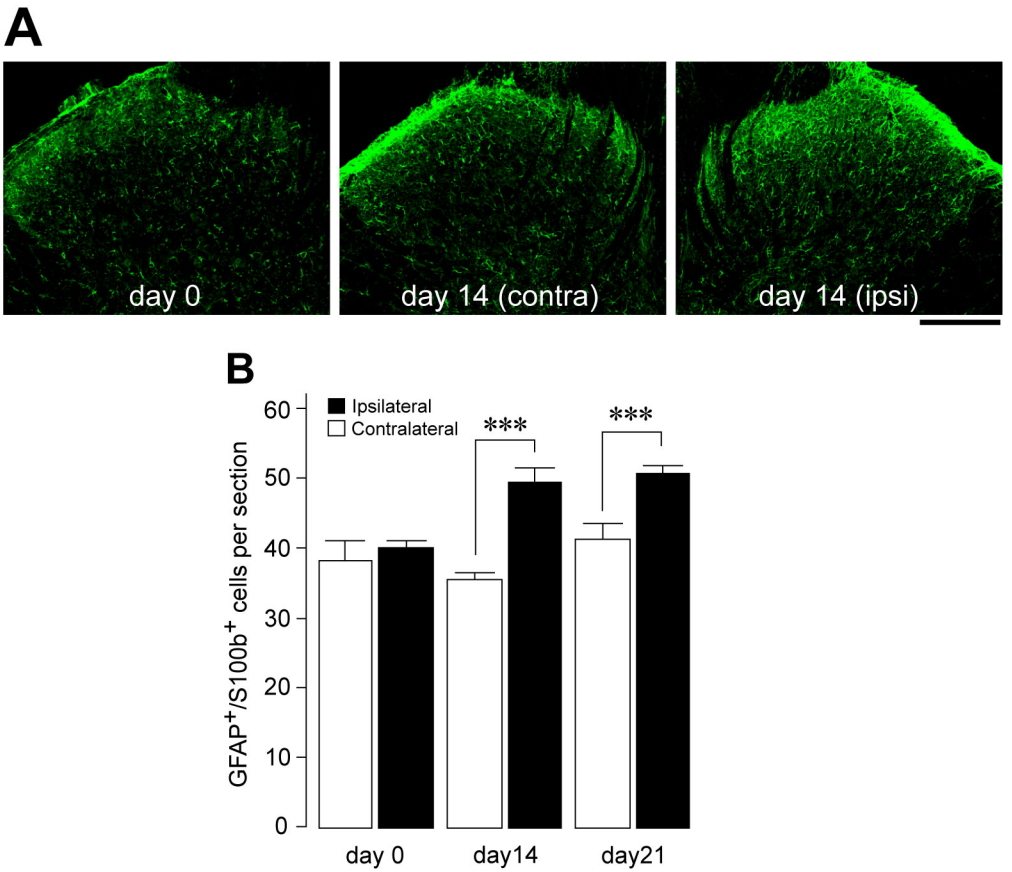

Supplementary Figure 1

(A) GFAP immunofluorescence in representative images of L5 dorsal horn sections from naive and PNI rats (day 14). Scale bar: 200 µm. (B) The numbers of GFAP+/S100β+ cells in the L5 dorsal horn ipsilateral and contralateral to PNI naive and PNI rats (days 14 and 21). Values represent the number of GFAP+/S100β+ cells (per section) (n=4 rats; ***P<0.001). Data are mean ± SEM.